NTP
National Toxicology Program

Multigenerational Reproductive Toxicology and Toxicology and Carcinogenesis Studies of Genistein in Sprague-Dawley Rats

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Outline

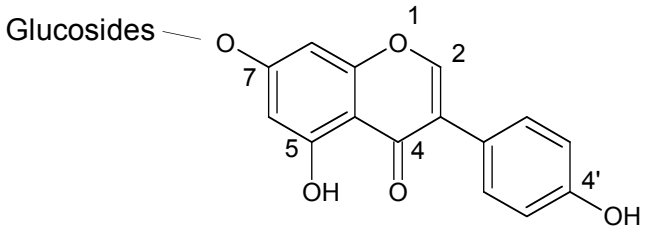
- Background on NTP's endocrine disruptor studies and genistein
- Summary of dose range finding study (TOX 79)
- Multigeneration study results (TR 539)
  - Exposure assessments (serum and tissue levels)
- Chronic study results (TR 545)

Background

- Endocrine Disruptor Evaluations Conducted Under NIEHS/NTP-FDA/NCTR Interagency Agreement
  - Evaluate long term effects of a series of compounds of varying potencies (genistein, nonylphenol, ethinyl estradiol, vinclozolin, methoxychlor)
  - Multigeneration reproductive studies with differing exposure windows across generations to evaluate the possibility of magnification of subtle effects across generations, reversibility
  - Include doses within likely human exposure range and/or below reported NOAEL
  - Chronic effects following exposure during different exposure windows
  - Original plan called for testing of pure compounds with consideration of subsequent testing more complex mixtures (e.g. soy extract, chlorinated hydrocarbons identified in human breast milk)

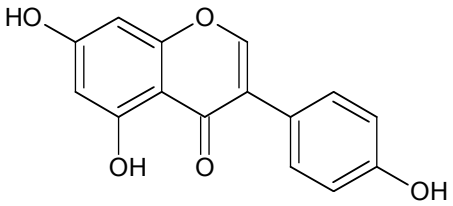
Background - Genistein

- Aglycone is metabolically derived from the glucoside, genistin
  - >95% of total isoflavones in glucoside form in cooked soybeans, texturized vegetable protein, soy milk
  - Fermented soy products, such as tofu and tempeh, contain approximately 20 and 40% aglycone
  - Generally, rodent and human studies indicate that oral genistin and genistein produce similar exposures to total and aglycone genistein
- Many proposed beneficial effects (soy and/or isoflavones)
  - Hormonally-induced cancers, cardiovascular disease, osteoporosis, adverse effects of menopause
- High exposures from soy infant formula, dietary supplements
- Weak estrogen with preference for ERβ
- Multiple other targets have been identified (e.g. topoisomerase II, tyrosine kinases, PPARs)



Glucoside Forms of Genistein in Soy
(Genistin, Acetylgenistin, Malonylgenistin)

Microbial and Intestinal
β-Glucosidases



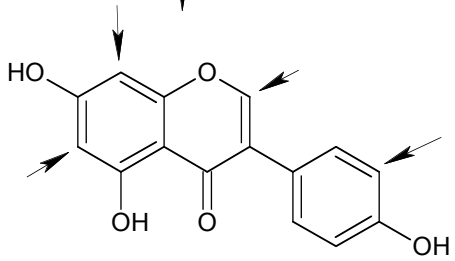
GENISTEIN

Microbial Reduction and
Ring Fission

Dihydrogenistein
6'-Hydroxy-O-desmethylangolensin
4-Hydroxyphenyl-2-propionic acid
4-Ethylphenol

Intestinal and Hepatic
UGT, SULT

Genistein 7 and 4'-glucuronides
Genistein 7'-sulfate



Hydroxylated Genistein Derivatives

Dose Range Finding Study Design

- Test Animal: CD (Sprague-Dawley) rat (NCTR colony)
- Exposure Window: GD 7 through PND 50/63/77
- Route of Exposure: Diet, Purina 5K96
- Control and 6 doses (0, 5, 25, 100, 250, 625, 1,250 ppm) for reproductive study, control and 4 doses for other endpoints
- Five litters per dose group, standardized litters (4 pups per sex)
- Goal: To select doses causing reproductive tract effects in pups that would not be likely to severely impair reproduction in the F_1 generation of the multigeneration study

Effects Observed in Dose Range Finding Study

(Delclos *et al*., Reprod. Tox. 15:1-17, 2001 and TOX 79)

Male mammary, hypertrophy/ hyperplasia	25 ppm/ 250 ppm
Male kidney, mineralization	250 ppm
Epididymis, hypospermina/aspermia	625/1250 ppm
Female mammary, hyperplasia	625 ppm
Vaginal dyssynchrony/ ovarian degeneration	625 ppm/ 1250 ppm
Retention/depletion of elongated spermatids	1250 ppm
Inflammation, dorsolateral prostate	1250 ppm
Acceleration of vaginal opening	1250 ppm
Body weight depression	1250 ppm

Other effects observed in the dose range finding study at 25 ppm and above

- Immunotoxicity study (K. White, D. Germolec)
  - increased anti-CD3-mediated splenocyte proliferation, M and F
    - Suggests potential for increased cell-mediated immunity
  - altered spleen T- and B-cell populations, M and F
  - decreased bone marrow subpopulations, M

- Thyroid peroxidase activity decreased (H. Chang, D. Doerge)

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Multigenerational Reproductive Toxicology Study of Genistein in Sprague-Dawley Rats

TR 539

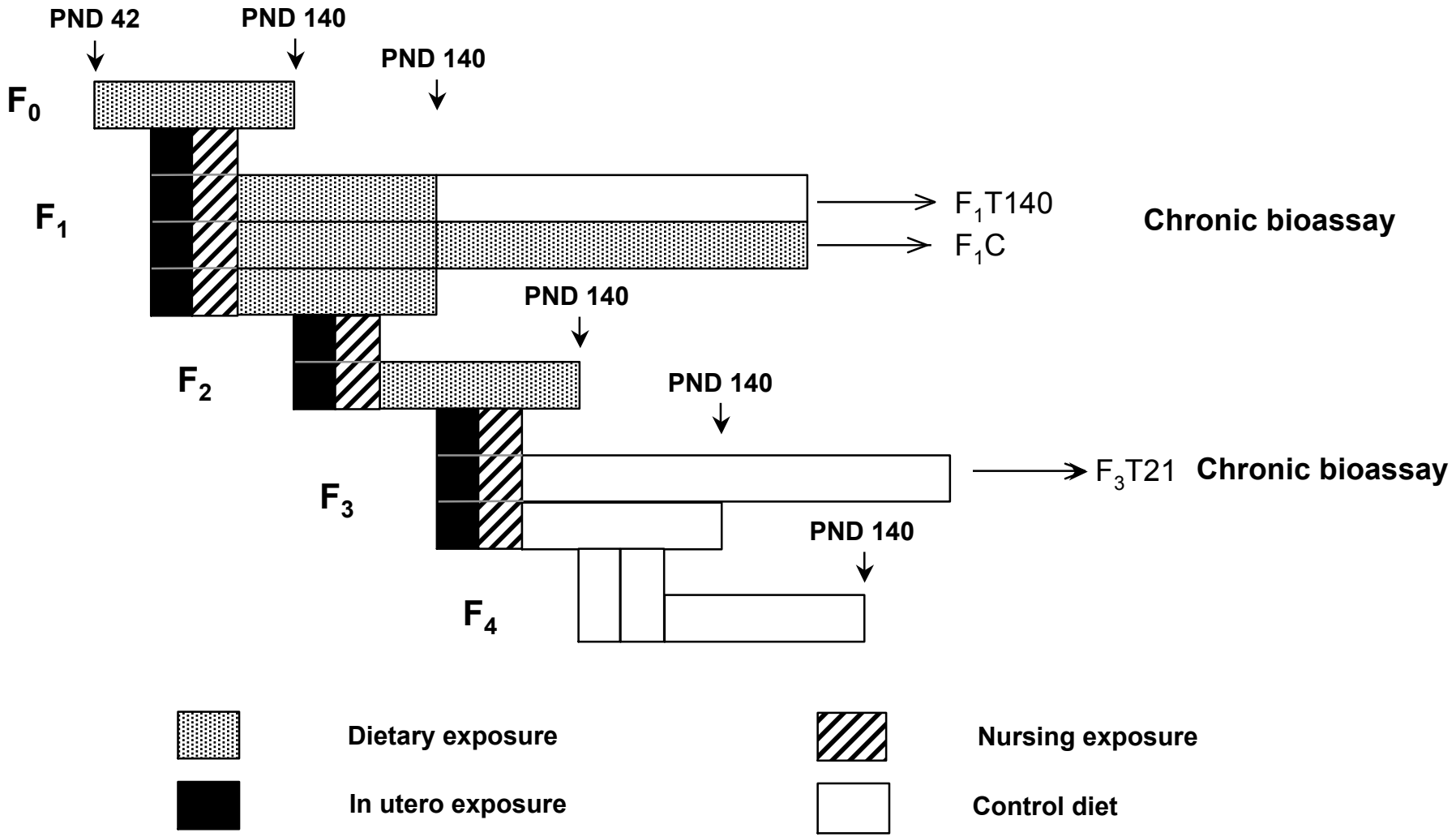
Multigeneration Study Design

- 0, 5, 100, and 500 ppm in Purina 5K96 (soy-, alfalfa-free) diet to NCTR CD (Sprague-Dawley) rats

- Dosing (reproductive phase, terminated PND 140 in all generations)
  - F_0: from 28 days prior to mating to PND 140
  - F_1, F_2: from conception to sacrifice at PND 140
  - F_3: from conception through weaning at PND 21
  - F_4: no exposure

- 35 or 40 (40 in F2 only) Breeding pairs per dose group, animals from 25 litters per dose group randomly selected for evaluation

Multigeneration Dosing Scheme



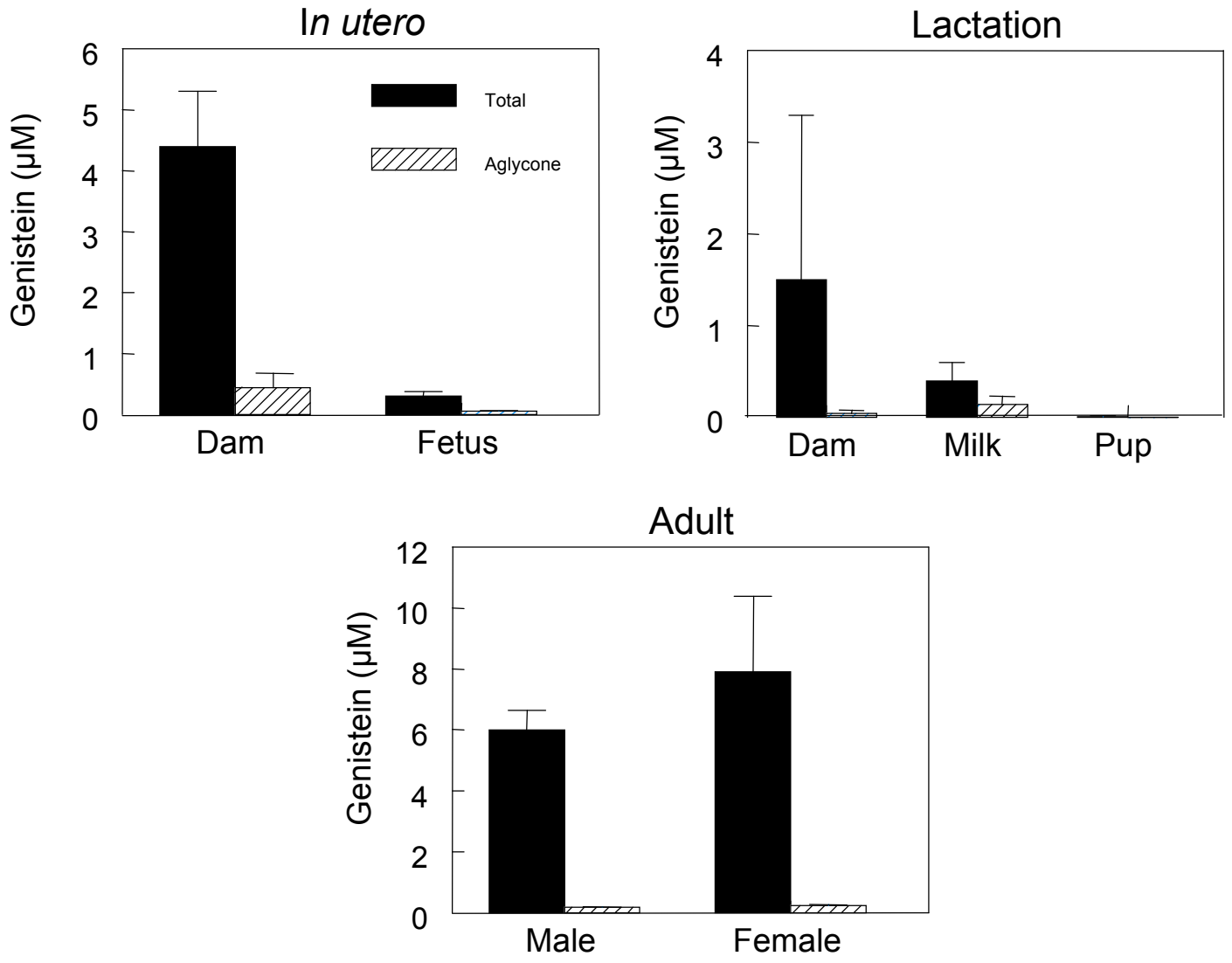
Tissue Distribution and Placental Transfer
(Chang *et al*., 2000 and Doerge *et al*., 2002)

- Concentrations of biologically active aglycone in tissues in nM-μM range and higher than in blood
- Fraction as aglycone higher in tissues vs. blood (up to 100% vs. 1-3%)
- Total genistein in fetal serum 20-50x lower than dams after oral administration
- Aglycone genistein in fetal serum 30-50% vs. 1-3% of total
- Fetal serum aglycone concentration more comparable to that in adults (2-5x lower)

Genistein Lactational Transfer

- 500 ppm, milk taken at PND 7, blood PND 10
- Efficient transfer of genistein aglycone into milk
- Low dose of genistein in milk vs. diet (40 ppb vs. 500 ppm)
- Low serum levels of genistein in pups
- Any developmental effects observed would result either from fetal exposures or very potent effects in neonatal period

Serum Genistein Levels During Various Exposure Phases
Measured at 500 ppm dose equivalent



Genistein Levels in Adult Rats: Relevance to Human Exposure Levels

(Chang *et al*., J. Nutr. 130: 1963-1970, 2000)

Dietary Concentration (ppm)	Ingested dose (mg/kg/day)	Serum Concentration (µM)	Human Relevance (Serum levels)
5	~0.4	0.06 – 0.1	Adults on typical Western diets
100	~8	0.6 – 0.9	Adults on typical Asian diet or dietary supplements
500	~40	6 - 8	Achievable in infants on soy formula

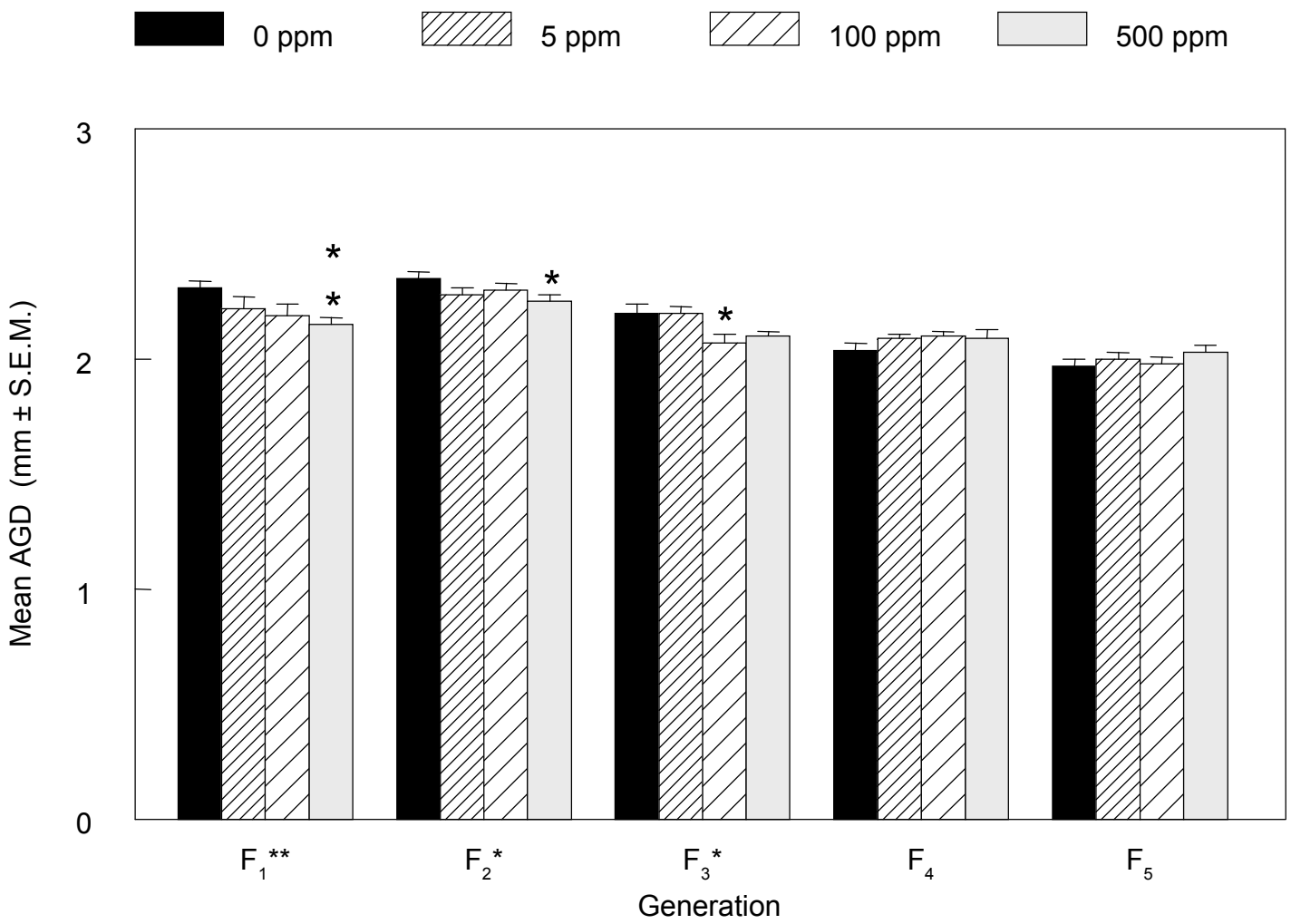
Body Weight and Food Consumption

Endpoint	Generation				
	F_0	F_1	F_2	F_3	F_4
Body Weight					
Females					
Preweaning	NA	↓ (500)	↓ (500)	↓ (500)	↓ (500)
Postweaning	↓ (500)	↓ (100, 500)	↓ (500)	-	↓ (500)
Males					
Preweaning	NA	↓ (5, 100, 500)	↓ (500)	↓ (500)	↓ (500)
Postweaning	-	↓ (100, 500)	-	-	-
Food Consumption					
Females	↓ (500)	↓ (500)	↓ (500)	-	↓ (500)
Males	-	-	-	-	-

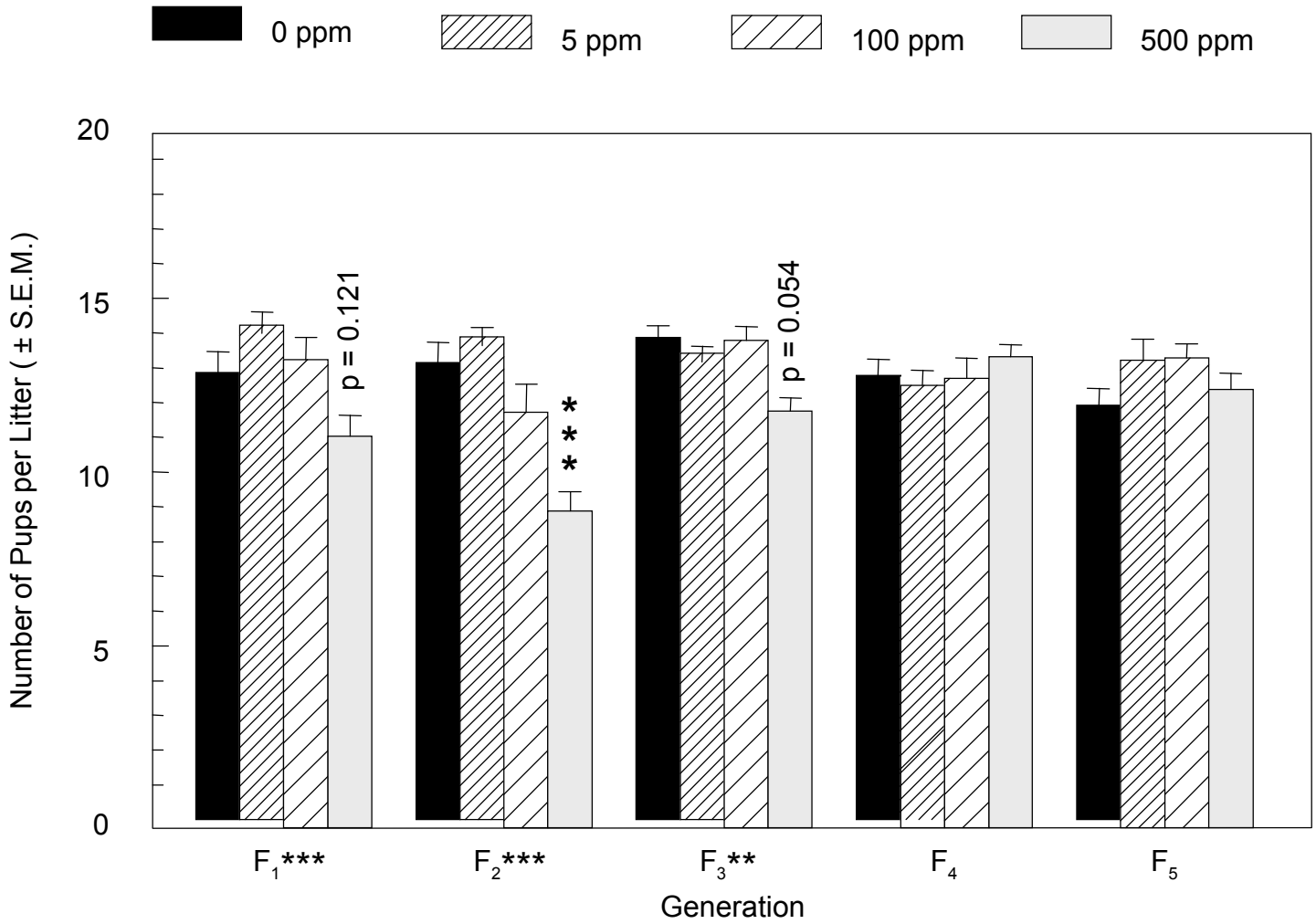
Genistein Multigeneration Study

Endpoint	F_0	F_1	F_2	F_3	F_4
Accelerated Vaginal Opening	NA	100 500 LT	500 LT	500 LT	LT
Aberrant/prolonged cycles 5 wks old	NA	500	500	-	-
Aberrant cycles 20 wks old	-	-	5 (% days E)	500	-
Decreased anogenital distance F, except F_1 (M and F)	NA	500 LT	500 LT	100 LT	-
Decreased litter size	NA	LT	500	LT	-
Renal tubule mineralization, Males PND 140	-	100 500 LT	100 500 LT	-	-
Male mammary hyperplasia PND 140	500	100 500 LT	100 500 LT	LT	-

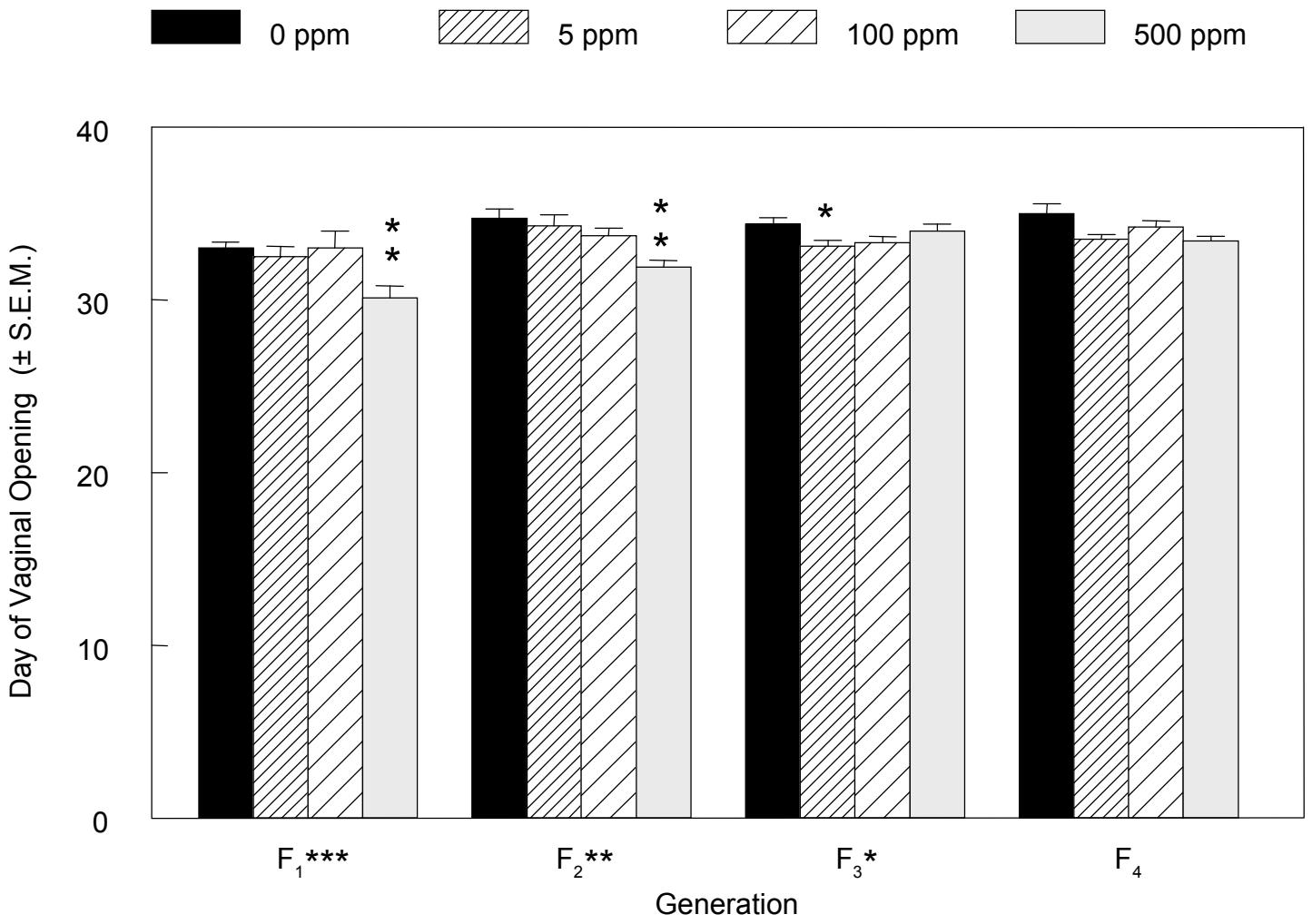
PND 2 Anogenital Distance (AGD) - Females



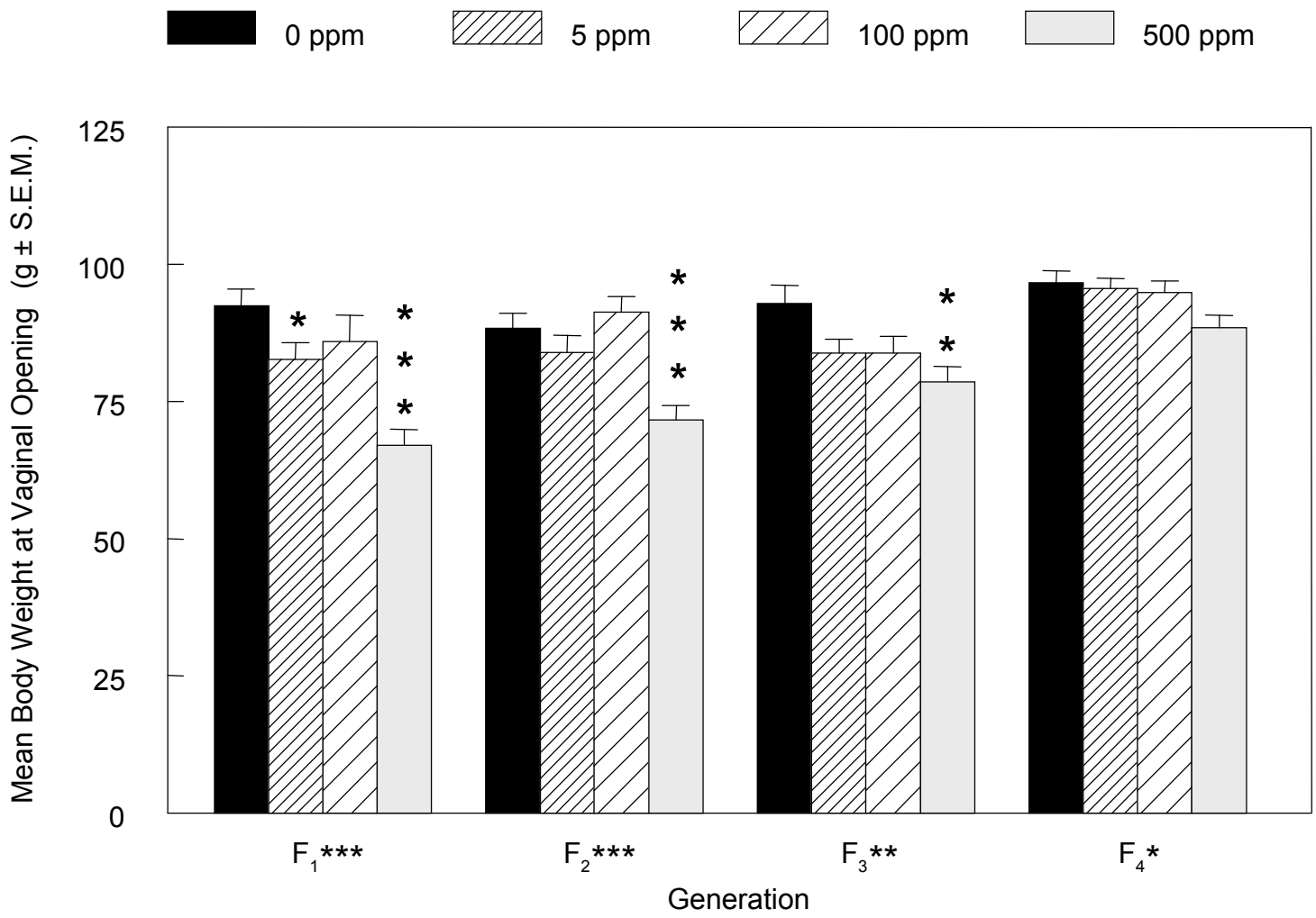
Litter Size


0 ppm
5 ppm
100 ppm
500 ppm
Number of Pups per Litter (± S.E.M.)
20
15
10
5
0
p = 0.121
p = 0.054
***
F1***
F2***
F3**
F4
F5
Generation

Age at Vaginal Opening



Body Weight at Vaginal Opening

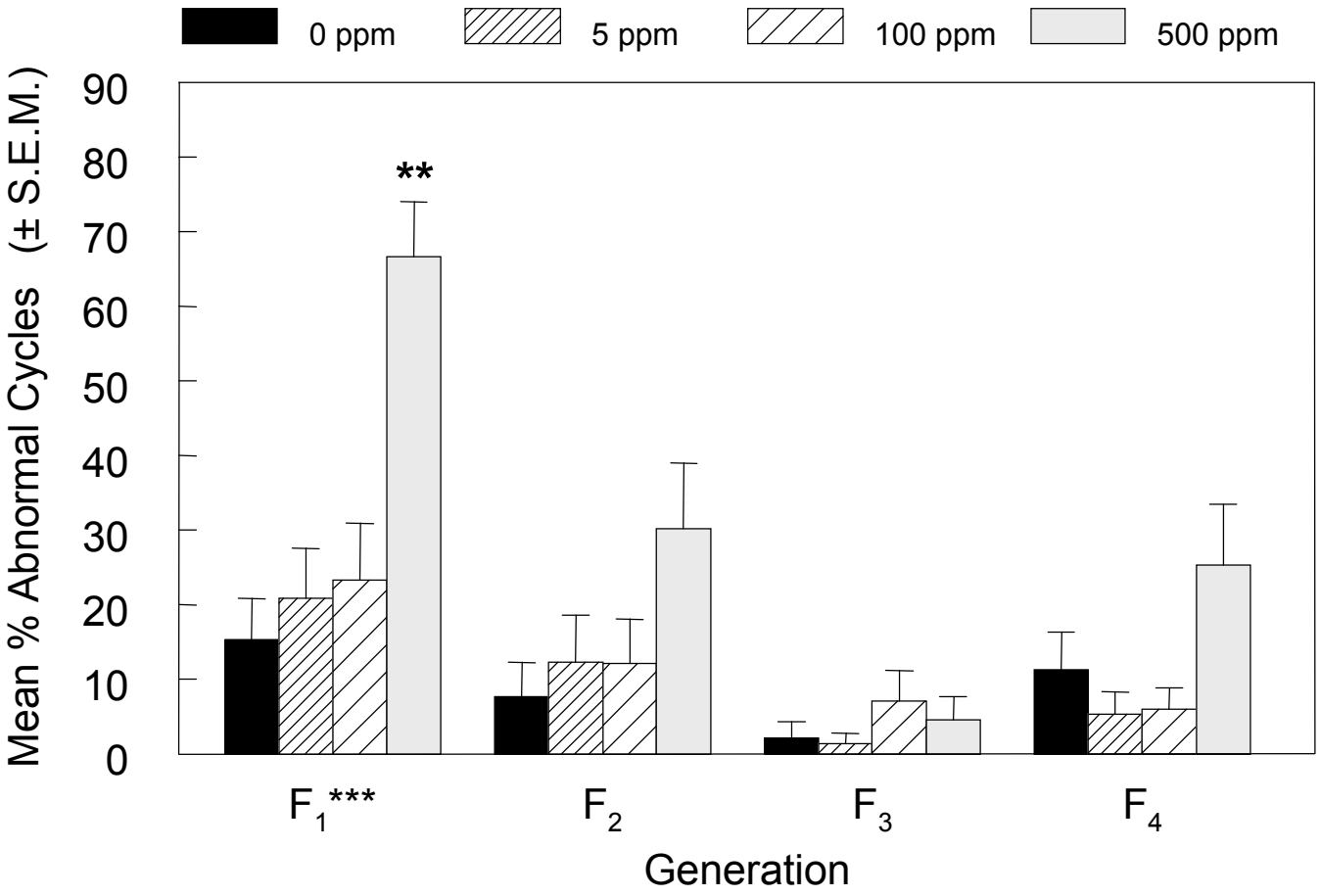


Vaginal Cytology Data from Genistein Multigeneration Study

- F_1-F_4: 14 consecutive days from 3 days after vaginal opening
- F_0-F_4: 10 consecutive days prior to necropsy (PND 130-140, breeders, after delivering and nursing litters)
- In subsequent slides, abnormal cycle defined as 4 or more days of diestrus or 3 or more days of estrus in a 5 day period

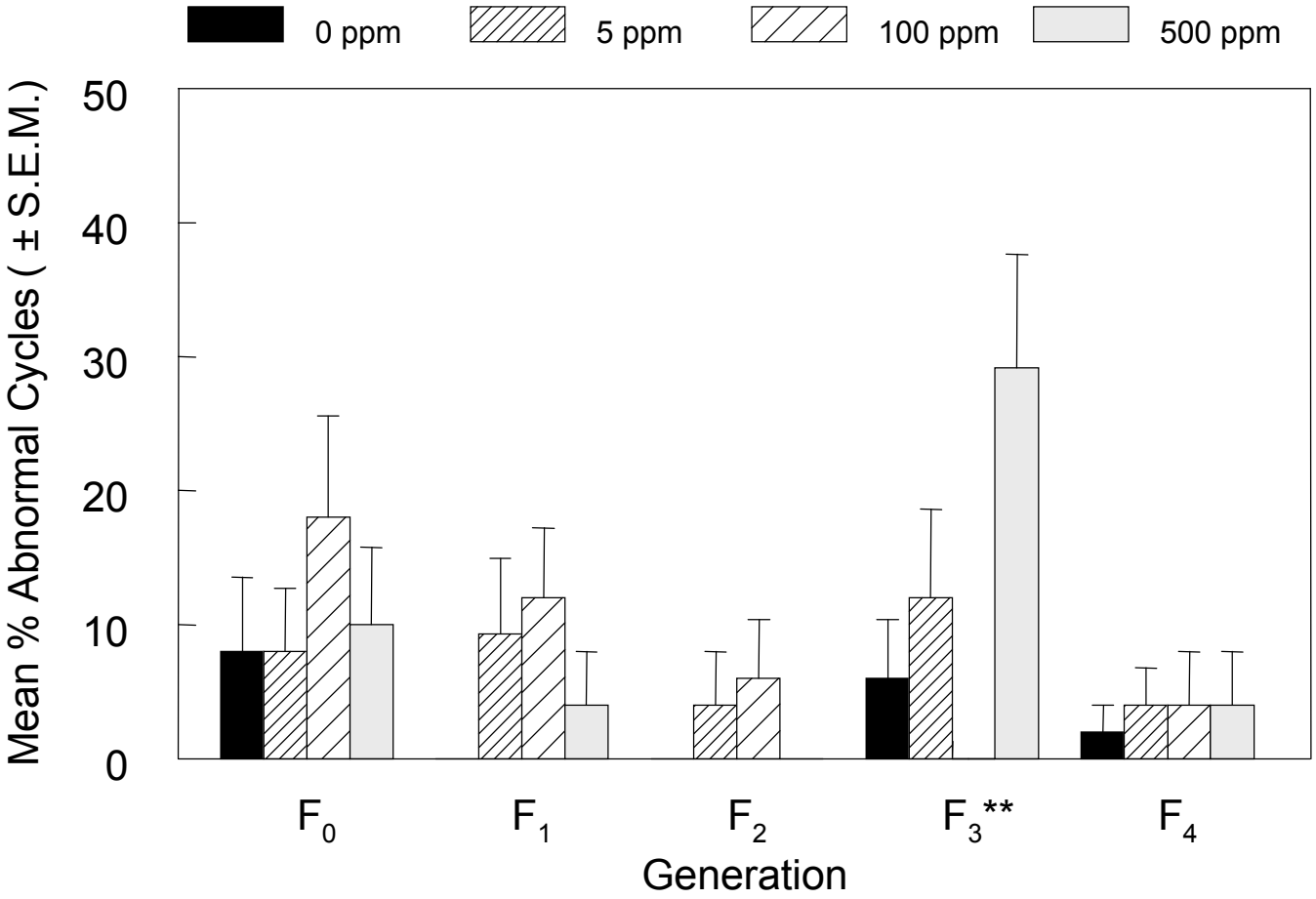
% Abnormal Cycles - Estrus and Diestrus

After Vaginal Opening

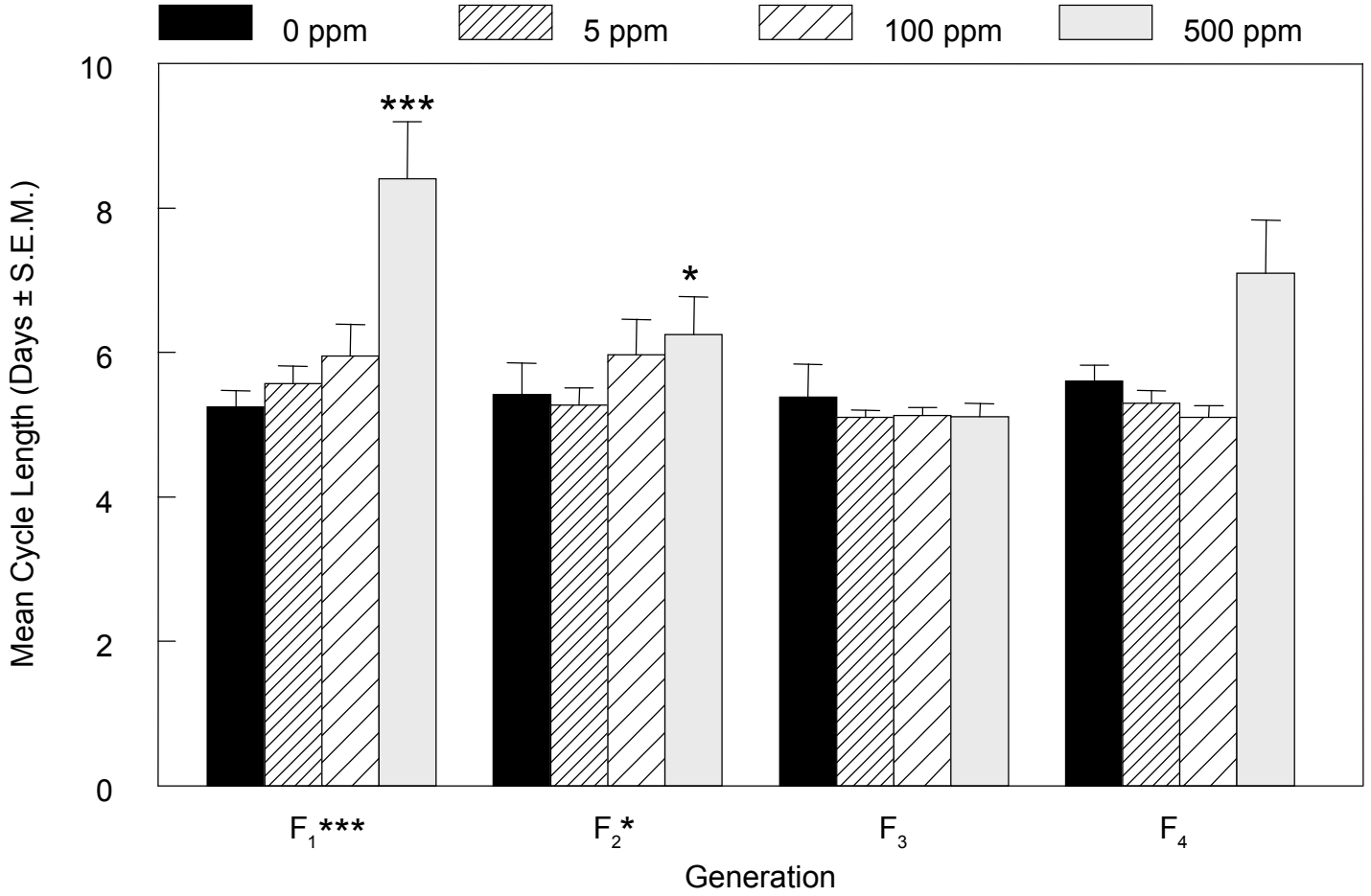


% Abnormal Cycles - Diestrus and Estrus

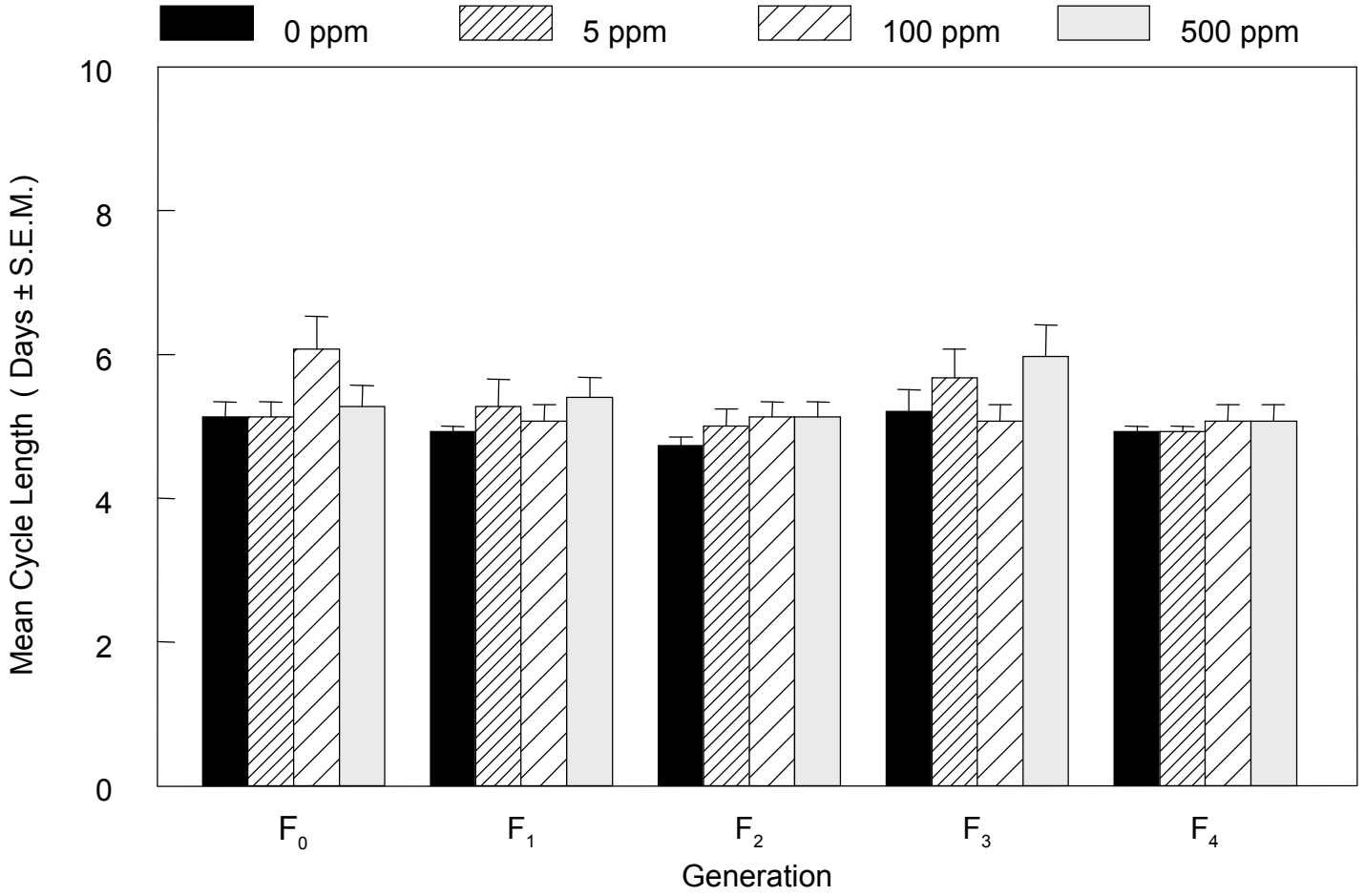
Before Sacrifice



Length of Cycle
After Vaginal Opening



Length of Cycle
Before Sacrifice



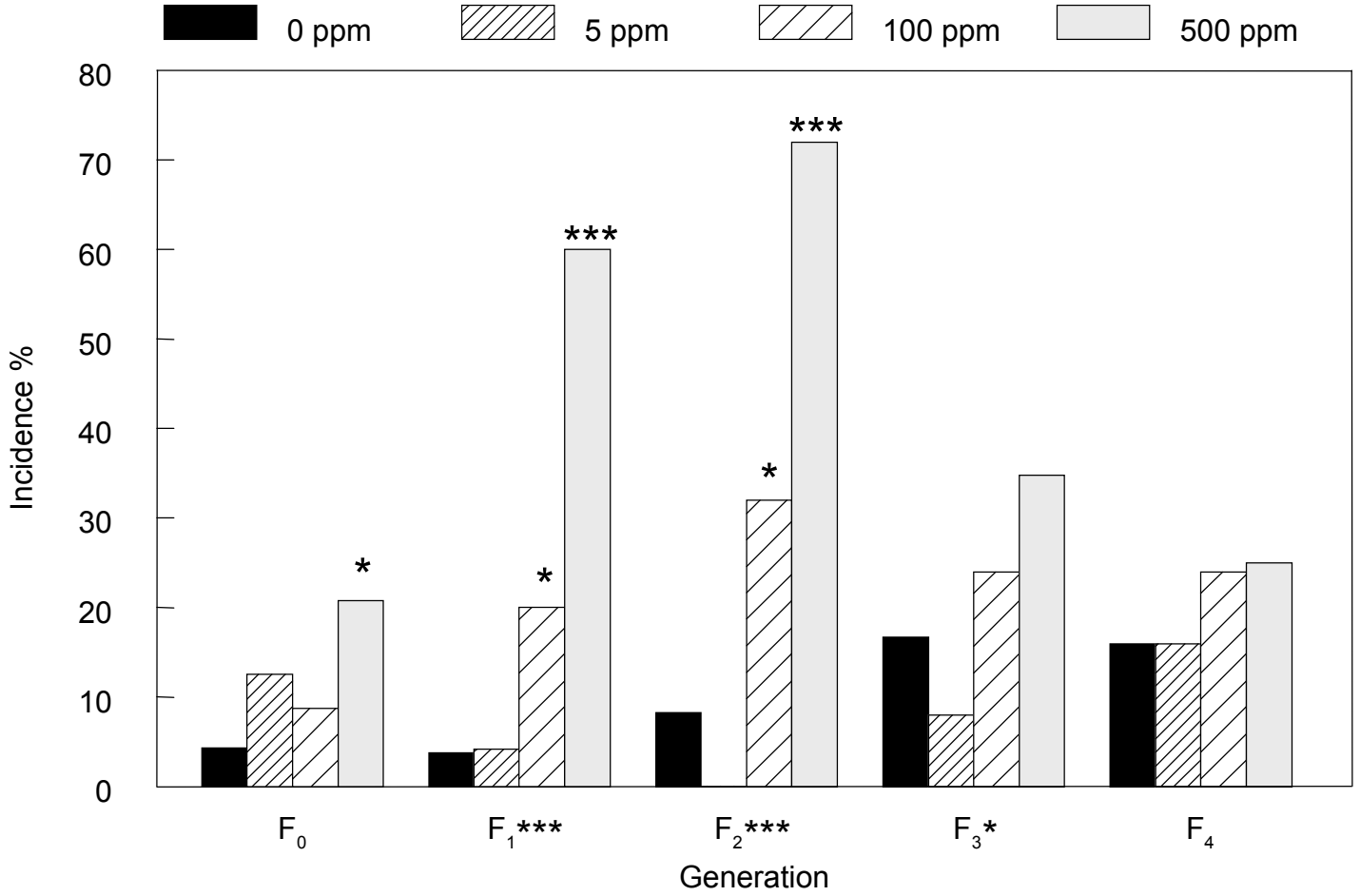
Male endpoints

- No effects on spermatogenesis
- No effects on markers of male puberty
- No effects on prostate weights or histology; no consistent effects on weights or histology of other male reproductive tract organs

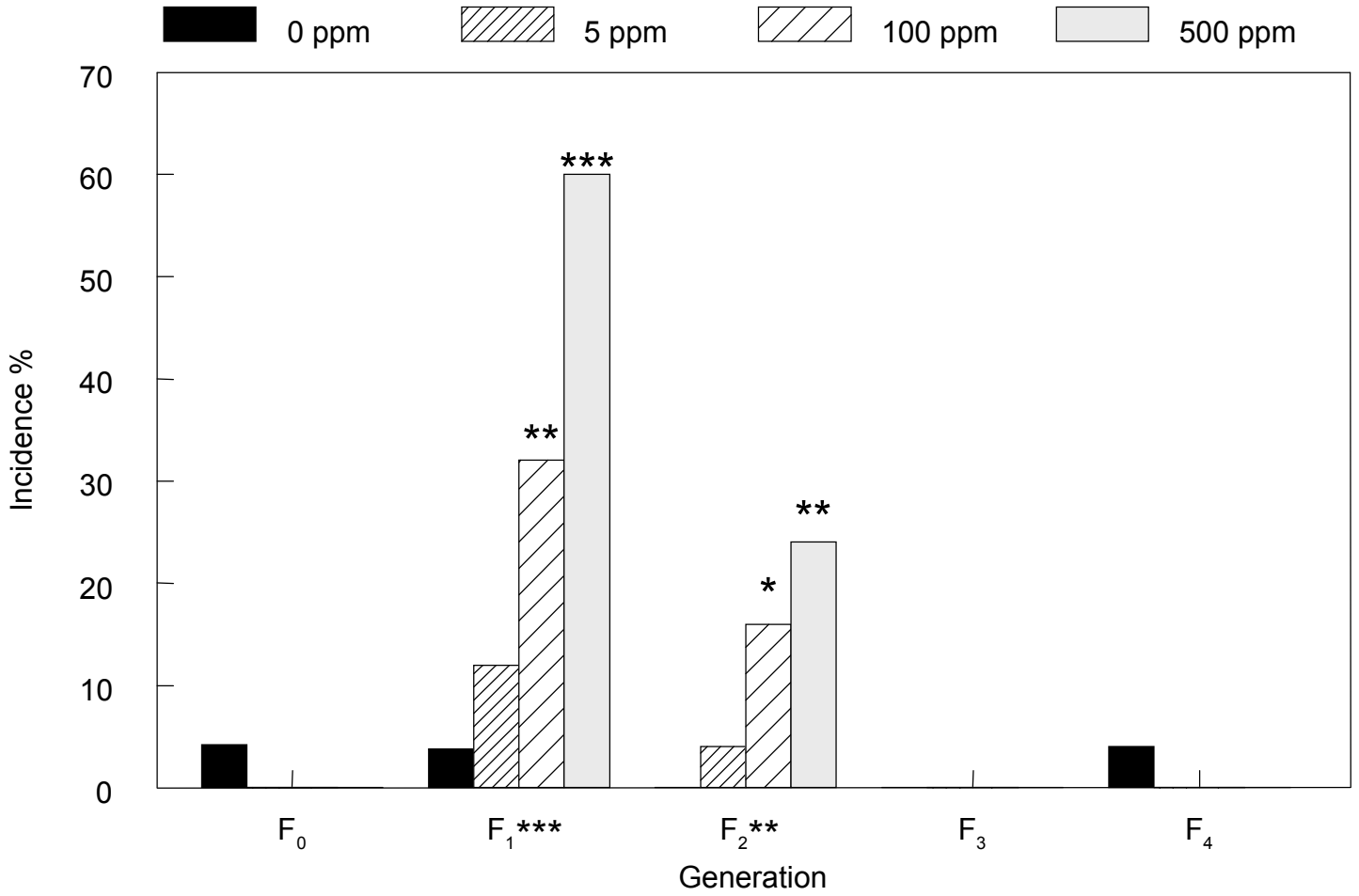
- Stimulation of male mammary gland hyperplasia and mineralization of renal tubules

Male Mammary Gland Hyperplasia

PND 140



Male Kidney Mineralization
PND 140



Conclusions (1): Multigeneration Study (TR 539)

- Reproductive toxicity evidenced by:
  - Reduced litter sizes (F_1, F_2, F_3, trends; F_2, 500 ppm vs control)
  - Accelerated time and/or lower body weights at vaginal opening (trends, 500 ppm vs control F_1, F_2, F_3)
  - Increased aberrant cycles and/or length of cycle (F_1, F_2 increasing trend, 500 ppm vs control)
  - Male mammary gland alveolar/ductal hyperplasia (500 ppm vs control, F_0-F_2; 100 and 500 ppm vs control, F_1 and F_2; trends, F_0-F_3)

Conclusions (2): Multigeneration Study (TR 539)

- Other effects
  - Increased renal tubule mineralization (males, 100 and 500 ppm, F_1 and F_2)
  - Decreased body weight gains, dependent on sex and generation
- Effects confined to, or more prominent for, continuously exposed generations
- With the exception of reduced body weight gain for pups of both sexes in preweaning period, no evidence for carryover or magnification of effects